

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

June 28, 2000

Honorable Assistant Commissioner for Patents  
Washington, D.C. 20231  
BOX: Patent Application

S I R:

Transmitted herewith for filing are the specification and claims of the patent application of:

Mercy M. Davidson for  
Inventor(s)

IMMORTALIZATION OF HUMAN POST-MITOTIC CELLS  
Title of Invention

Also enclosed are:

x 5 sheet(s) of      informal x formal drawings.

x Oath or declaration of Applicant(s). (unsigned)

X A power of attorney (unsigned)

     An assignment of the invention to                                     

     A Preliminary Amendment

X A verified statement to establish small entity status under 37 C.F.R. §1.9 and §1.27.

The filing fee is calculated as follows:

CLAIMS AS FILED, LESS ANY CLAIMS CANCELLED BY AMENDMENT

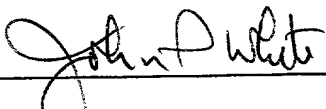
	NUMBER FILED		NUMBER EXTRA*		RATE		FEE	
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Total Claims	19 -20	=	0	X	\$ 9.00	\$18.00	= \$ 0	\$
Independent Claims	5 -3	=	2	X	\$39.00	\$78.00	= \$ 78	\$
Multiple Dependent Claims Presented: <u>    </u> Yes <u>    </u> No					\$130.00	\$260.00	= \$ 130	\$
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Applicant: Mercy M. Davidson  
Serial No.: Not Known Yet  
Docket No.: 56614  
Attorney's: JPW/JML/HA  
Filed: June 28, 2000

- ☒ A check in the amount of \$ 582.00 to cover the filing fee.
- ☐ Please charge Deposit Account No. \_\_\_\_\_ in the amount of \$ \_\_\_\_\_.
- ☒ The Commissioner is hereby authorized to charge any additional fees which may be required in connection with the following or credit any over-payment to Account No. 03-3125:
- ☒ Filing fees under 37 C.F.R. \$1.16.
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- ☐ The issue fee set in 37 C.F.R. \$1.18 at or before mailing of the Notice of Allowance, pursuant to 37 C.F.R. \$1.311(b).
- ☒ Three copies of this sheet are enclosed.
- ☐ A certified copy of previously filed foreign application No. \_\_\_\_\_ filed in \_\_\_\_\_ on \_\_\_\_\_.
- Applicant(s) hereby claim priority based upon this aforementioned foreign application under 35 U.S.C. \$119.
- ☒ Other (identify) Express Mail Certificate, bearing label No. EE474772039US and one loose set of drawings.

Respectfully submitted,

  
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Serial or Patent No.: Not Known Yet Docket No: 56614  
Filed or Issued: June 28, 2000  
Title of Invention or Patent: IMMORTALIZATION OF HUMAN POST-MITOTIC CELLS

VERIFIED STATEMENT (DECLARATION) CLAIMING  
SMALL ENTITY STATUS UNDER 37 C.F.R. §1.9(f)  
AND §1.27(d) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of Organization: The Trustees of Columbia University in  
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Address of Organization: Broadway and West 116th Street  
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TYPE OF ORGANIZATION:

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I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 C.F.R. §1.9(e)\* for purposes of paying reduced fees under 35 U.S.C. §41(a) and 41(b), with regard to the invention entitled  
IMMORTALIZATION OF HUMAN POST-MITOTIC CELLS

by inventor(s) Mercy M. Davidson  
described in:

☒ the specification filed herewith  
☐ application serial no. \_\_\_\_\_ filed \_\_\_\_\_  
☐ patent no. \_\_\_\_\_ issued \_\_\_\_\_

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive each individual, concern, or organization known to have rights to the invention is listed below<sup>a</sup> and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 C.F.R. §1.9(d)\* or a nonprofit organization under 37 C.F.R. 1.9(e)\*

<sup>a</sup>NOTE: Separate verified statements are required from each person, concern, or organization having rights to the invention averring to their status as small entities. 37 C.F.R. §1.27.

Name: \_\_\_\_\_  
Address: \_\_\_\_\_

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

Small Entity/Nonprofit  
Page -2-

Applicant: Mercy M. Davidson  
Serial No.: Not Known Yet  
Docket No.: 56614  
Attorney's: JPW/JML/HA  
Filed: June 28, 2000

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. 37 C.F.R. §1.28(b)\*.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing: Mr. Jack M. Granowitz  
Title In Organization: Executive Director, Columbia Innovation Enterprise  
Address: Columbia University, Engineering Terrace - Suite 363  
West 120th Street and Amsterdam, New York, New York 10027  
Signature: *Jack M. Granowitz*  
Date Of Signature: 6/21/00

\*See Reverse

008290-92470360

**Application  
for  
United States Letters Patent**

**To all whom it may concern:**

*Be it known that*

Mercy M. Davidson

*have invented certain new and useful improvements in*

IMMORTALIZATION OF HUMAN POST-MITOTIC CELLS

*of which the following is a full, clear and exact description*

---

**IMMORTALIZATION OF HUMAN POST-MITOTIC CELLS**

The invention was made in part with government funds under Grant Nos. HD 32062 and NS 11766 from the National Institutes of Health, U.S. Department of Health and Human Services. Therefore, the U.S. Government has certain rights in the invention.

Throughout this application, various publications are referenced by author's last name and year published and a listing of those references following the Experimental Details Section. The disclosures of these publications in their entireties are hereby incorporated by reference in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

**Background of the Invention**

Cardiomyocytes have a finite life-span in culture before becoming terminally differentiated. Attempts to immortalize ventricular cardiomyocytes in order to establish a cell line that can proliferate in culture have not been successful. Recently, Claycomb et al (1998) described a mouse atrial cardiomyocyte cell line, HL-1, which can be passaged serially, differentiate, and maintain the characteristics of adult mouse cardiomyocytes. This cell line could be a useful tool for cardiovascular research, but a mouse cell line can not answer questions that are specific to the human system. Currently there is no immortalized human cardiomyocyte cell line that can proliferate and differentiate in culture and can express the adult cardiomyocyte phenotype when culture conditions are manipulated. Using an unexpected, novel and unique mitochondrial function-based method to immortalize human primary cardiomyocytes from both adult and fetal heart tissue the cell lines described in this report have been passaged for over 100 generations and can be regrown from frozen stocks. The

cells express both cardiac and skeletal muscle-specific markers and can be induced to differentiate in culture to express adult skeletal muscle and cardiac muscle phenotypes.

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Summary of the Invention

5 The present invention provides an immortalized human  
cardiomyocyte cell line and an immortalized human  
vascular smooth muscle cell line. The present invention  
further provides a method for preparing a human  
immortalized cell line derived from a post-mitotic  
10 primary cell culture which comprises: (a) providing a  
cell culture of human primary post-mitotic cells, (b)  
providing a human fibroblast cell line which: (i) has  
been transfected with a replicable nucleic acid vector  
which immortalizes the fibroblast cell line, (ii) has  
15 been depleted of its mitochondrial DNA thereby rendering  
the fibroblast cell line subject to growth selection due  
incapacity to perform glycolysis; (c) co-culturing the  
human fibroblast cell line of step (b) with the cell  
culture of step (a) under appropriate conditions so that  
cell fusion occurs; and (d) growing the fused cells from  
20 step (c) in a selection medium which selects for cells  
with mitochondrial DNA, (e) selecting cells from step  
(d) which contain a nucleus which originated from the  
cells of the primary culture, so as to prepare the human  
immortalized cell line.

25 The present invention provides a human cardiomyocyte  
cell line designated AC10 (ATCC Patent Deposit  
Designation No. PTA-1501), cell line designated AC16  
(ATCC Patent Deposit Designation No. PTA-1500) and cell  
30 line designated RL14 (ATCC Patent Deposit Designation  
No. PTA-1499) derived from nonproliferating primary  
culture. Additionally, the present invention provides  
that the cells are human cells.



**Brief Description of the Figures**

**Figure 1:** Schematic diagram to illustrate generation of immortalized human cardiomyocyte cell lines.

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**Figures 2A-2D:** Immunocytochemical staining of cardiomyocyte cell line AC16 with monoclonal antibodies to SV-40 large T-antigen (Fig. 2A);  $\beta$ -myosin heavy chain (Fig. 2B); desmin (Fig. 2C), and connexin-43 (Fig. 2D).

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**Figure 3:** RT-PCR of cardiomyocyte clones (CM) showing,  $\beta$ -myosin heavy chain mRNA, and COX subunit VIIc mRNA. Lane 1 adult human heart, 2-5 CM clones, 6 human fibroblasts. M=DNA size marker.

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**Figures 4A-4B:** Vascular smooth cells stained with monoclonal antibodies to SV-40 large T-antigen (Fig. 4A) and succinate dehydrogenase (SDH) histochemistry (Fig. 4B).

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**Detailed Description of the Invention**

5 The present invention provides a human cardiomyocyte cell line designated AC10 (ATCC Patent Deposit Designation No. PTA-1501), cell line designated AC16 (ATCC Patent Deposit Designation No. PTA-1500) and cell line designated RL14 (ATCC Patent Deposit Designation No. PTA-1499) derived from nonproliferating primary culture. As used herein the term "nonproliferating primary cultures" encompasses cell cultures which become senescent after 2-3 passages (limited passage) and post-mitotic cells in culture. Such cultures also include those cells in culture that have exited the cell cycle and are no longer capable of undergoing mitosis (post-mitotic).

15 As used herein, the term "primary cultures" encompasses cells in culture that have been taken for an organism and not passaged. Primary cultures herein comprise but are not limited to cells in culture originally taken from vascular smooth muscle, skeletal myoblasts, neuronal cells, bone cells (osteoblasts, osteocytes), chondrocytes, normal cardiomyocytes.

25 AC10 cell line (ATCC Patent Deposit Designation No. PTA-1501), AC16 cell line (ATCC Patent Deposit Designation No. PTA-1500) and RL14 cell line (ATCC Patent Deposit Designation No. PTA-1499) were received on March 16, 2000 and were accepted by the American Type Culture Collection (ATCC) 10801 University Blvd., Manassas, VA 20110-2209, which is an International Depository Authority recognized under the provisions of the Budapest Treaty. All restrictions upon public access to these deposits will be irrevocably removed upon the grant of a patent on the subject application. The deposit will be replaced if viable samples cannot be dispensed by the ATCC.

40 The present invention provides an immortalized human cardiomyocyte cell line. This cell line may be derived

from a post-mitotic human cardiomyocyte cell culture.

In one embodiment, the post-mitotic cell line is a cardiomyocyte cell line. In another embodiment, the post-mitotic cell line is a vascular smooth muscle cell line. In one embodiment, the post-mitotic cell line is a neuronal cell line. In one embodiment, the post-mitotic cell line is a skeletal myoblast cell line.

The present invention provides for an immortalized human cardiomyocyte cell line and an immortalized human vascular smooth muscle cell line.

In one embodiment, the cell line integrates functionally with normal or myopathic cardiac tissue as determined by measurement of syncytial beating of the tissue. This syncytial beating can be easily measured in cell culture.

The present invention provides a method for treating damaged cardiac tissue in a subject which comprises transplanting an immortalized human cardiomyocyte cell line of the present invention into a subject's heart containing damaged cardiac tissue.

The present invention provides for a method for preparing a human immortalized cell line derived from a post-mitotic primary cell culture which comprises: (a) providing a cell culture of human primary post-mitotic cells, (b) providing a human fibroblast cell line which: (i) has been transfected with a replicable nucleic acid vector which immortalizes the fibroblast cell line, (ii) has been depleted of its mitochondrial DNA thereby rendering the fibroblast cell line subject to growth selection due incapacity to perform glycolysis; (c) co-culturing the human fibroblast cell line of step (b) with the cell culture of step (a) under appropriate conditions so that cell fusion occurs; and (d) growing the fused cells from step (c) in a selection medium which selects for cells with mitochondrial DNA, (e)

5 In one embodiment, the cell culture of human primary non-proliferating cells in step (a) is a cell culture of primary human cardiac cells, primary human skeletal myoblast cells, human neuronal cells, or primary human osteoblast cells.

In another embodiment of the invention, the fibroblast  
15 cell line is designated DWFb1.

The present invention provides for a method for determining whether a composition of matter inhibits cardiomyocyte cell function which comprises: (a) admixing the composition with cells of an immortalized cardiomyocyte cell line prepared by the method for preparing a human immortalized cell line of the present invention, in cell culture; and (b) determining whether the cells in step (a) exhibit normal cardiomyocyte cell function by measuring gene expression or by measuring syncytial beating in culture, wherein decreased cardiomyocyte cell function indicates that the composition inhibits cardiomyocyte cell function.

The present invention also provides for a method for  
35 determining whether a composition of matter enhances  
cardiomyocyte cell function which comprises: (a)  
admixing the composition with cells of an immortalized  
cardiomyocyte cell line prepared by the method of  
preparing an immortalized human cardiomyocyte cell line  
40 of the present invention, in cell culture; and (b)

determining whether the cells in step (a) exhibit normal cardiomyocyte cell function by measuring gene expression or by measuring syncytial beating in culture, wherein increased cardiomyocyte cell function indicates that the composition enhances cardiomyocyte cell function.

In one embodiment, the composition of matter is a peptide or a peptidomimetic. In another embodiment, the composition of matter is a small organic molecule. In another embodiment, the composition of matter is a nucleic acid. In another embodiment, the composition of matter is associated with a pharmaceutically acceptable carrier.

In one embodiment, the carrier is a diluent, an aerosol, a topical carrier, an aqueous solution, an ionic solution, a nonaqueous solution or a solid support.

The selection of cells with a nucleus which originated from cells of the primary culture may be carried out in several ways. For example, one may be able to select against the growth of fibroblast cells in culture by adding to the culture antibodies specific for cell surface determinants on the fibroblasts and then adding complement to fix the cells, thereby stopping the growth of these fibroblast cells and selecting for growth of cells which have the nucleus of the cells of the primary cell cultures. Another way to so select is to screen for the expression of a gene which is specific for cells of the primary culture and which the fibroblast cells would not express. Then, one would subclone the cells which are positively expressing the specific genes and therefore would obtain a pure culture of cells which have a nucleus which originates from the primary cell culture.

In one embodiment of the invention, the cell culture of human primary non-proliferating cells of step (a) is a primary human cardiac cell culture, a primary human skeletal myoblast cell culture, a human neuronal cell

culture, or a primary human osteoblast cell culture.

In another embodiment, the replicable vector is an SV-40 mammalian vector. In another embodiment of the invention, the fibroblast cell line is designated DWFb1.

In one embodiment, the pharmaceutical composition is a peptide or a peptidomimetic. In one embodiment, the pharmaceutical composition is a small organic molecule. In one embodiment, the pharmaceutical composition is a nucleic acid. In one embodiment, the pharmaceutical composition is bound to a pharmaceutically acceptable carrier. In one embodiment, the carrier is a diluent, an aerosol, a topical carrier, an aqueous solution, an ionic solution, a nonaqueous solution or a solid support.

As used herein, "mutant DNA" encompasses but is not limited to chromosomal defects (eg, trisomy 13, trisomy 18) or genetic diseases (eg, trisomy 21, Turner's syndrome [XO], Holt-Oram syndrome).

As used herein, "foreign" encompasses a DNA or RNA not in the genus of the tissue or cell.

As used herein the term "immortalize" encompasses the process of whereby a cell line can be passaged indefinitely in culture, while the cells in culture retain the functions and features of the primary cells in the culture the day the culture was begun. In one embodiment cells were immortalized by transferring the SV-40 gene into the cells of the primary culture.

As used herein, the term "primary cells" encompasses cells derived from the original tissue as obtained and manipulated to generate primary cultures.

As used herein,  $\rho^0$  represents cells of the cell line rendered mtDNA-less (a cell line wherein the mitochondrial DNA of the cells is depleted).

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As used herein, "post-mitotic cells" encompass cells that are no longer dividing or undergoing mitosis.

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As used herein, "screening" denotes immunocytochemical evaluation of cell specific markers.

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from myocardial DNA mutants.

In another embodiment the primary cultures are derived from adult human tissue or from fetal human tissue.

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In another embodiment the primary cultures are derived from cells that have exited the cell cycle or are post-mitotic.

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The invention also provides a cell line derived from non-proliferating primary cultures.

In yet another embodiment the cells express  $\beta$ -myosin heavy chain and connexin-43.

15

Further, the present invention provides cardiomyocyte cells which when transplanted to the heart can integrate functionally forming a syncitium with normal or myopathic cardiac tissue.

20

The present invention provides a method for identifying a pharmaceutical preparation which is capable of enhancing or inhibiting cardiomyocyte cell growth comprising: (a) exposing the human cardiomyocyte cell line derived from nonproliferating primary cell cultures to a pharmaceutical preparation; (b) evaluating the mitochondrial function of the cell line; and (c) evaluating physiological and pathological changes of the cardiomyocyte function.

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As used herein, the term "evaluating" encompasses determining the biochemical, electrophysiological or molecular genetic composition of a cell. The biochemical, electrophysiological or molecular genetics of a cell line is determined by experimental procedures described herein comprising but not limited to immunocytochemical screening, histochemistry, molecular genetic analyses and electrophysiological studies. These techniques are known by persons skilled in the art.

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Further, the invention provides a method of identifying cardiomyopathy comprising: (a) evaluating the cell line derived from nonproliferating primary cell cultures; (b) replacing the mitochondrial DNA of the cell line in step  
5 (a) with mutant DNA; (c) evaluating the cells of step (b); and (d) comparing the evaluation of step (a) to that of step (b).

Finally, the invention provides a method of identifying  
10 nuclear mitochondrial DNA interactions comprising: (a) evaluating the cell line derived from nonproliferating primary cell cultures; (b) replacing the mitochondrial DNA of the cell line in step (a) with mutant DNA; (c)  
15 evaluating the cells of step (b); and (d) comparing the evaluation of step (a) to that of step (c).

As used herein, the term "pharmaceutical carrier" encompasses any of the standard pharmaceutical carriers such as phosphate buffered saline solution, water,  
20 emulsions such as oil/water emulsion or a triglyceride emulsion, various types of wetting agents, tablets, coated tablets and capsules. Typically such carriers contain excipients such as starch, milk, sugar, certain  
25 types of clay, gelatin, stearic acid, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients.

In the practice of any of the methods of the invention  
30 or preparation of any pharmaceutical preparation the effective amount administered will vary with the condition under investigation. For the purpose of this invention, the doses selected are known to one of skill in the art.

35 As used herein, characteristic features of cardiomyocytes are determined by immunocytochemical screening, histochemistry, molecular genetic analyses and electrophysiological studies.

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## Experimental Details

### Example 1: Human cardiomyocyte cell line

5 Human cardiomyocyte cell lines obtained from  
non-proliferating primary cultures derived from both  
adult and fetal heart tissues using an unexpected, novel  
and unique method that may be applied to all primary  
cultures that have exited the cell cycle was established  
10 by this invention. The cell lines express both cardiac  
and skeletal muscle-specific genes. Presented in this  
invention is the only human cardiomyocyte cell line  
currently available. The transformed cardiomyocytes  
express  $\beta$ -myosin heavy chain and connexin-43. The presence of  
15 gap junctions and the major cardiac-specific gap  
junction protein, connexin-43 will allow the cells to  
form a syncytium and to integrate functionally with  
normal or myopathic cardiac tissue. Additionally, these  
cardiomyocyte cell lines are useful tools for evaluating  
20 pharmacological, electrophysiological, and biochemical  
effects of pharmaceutical preparations. These cell lines  
are potentially invaluable *in vitro* models to study  
differentiation of muscle in normal and pathological  
states with wide applicability in cardiovascular and  
25 neuromuscular research. In addition, by methods  
described previously (King, et al.), it is possible to  
deplete the cardiomyocyte cell lines of their endogenous  
mtDNA content and repopulate them with mtDNA containing  
mutations commonly associated with maternally inherited  
30 cardiomyopathies thus providing an excellent *in vitro*  
model to study the tissue specific phenotype that may  
segregate with the respective mutation.

## **MATERIALS AND METHODS**

### *Primary cultures*

35 Adult ventricular heart tissue was obtained from the  
heart transplantation facility and fetal heart was  
obtained from the Department of Obstetrics and  
Gynecology, Columbia University, (IRB#0534) (ventricular  
40 heart tissue is illustrative as described herein,

subsequent experiments used the same method with  
different tissue). In later experiments human tissue was  
obtained from the same facility and used to establish  
primary cell cultures of vascular smooth muscle, skeletal  
5 myoblasts, neuronal cells or cardiomyocytes with normal  
or pathological myocardial DNA (mutants.) The  
ventricular tissue was dissected and minced under a  
dissection microscope. The tissue was transferred to a  
glass chamber and extensively trypsinized at 37° C. The  
10 enzymatically dissociated cells consisting of a mixture  
of all the constituent cell types of cardiac tissue were  
resuspended in DMEM F-12, supplemented with 12.5% Fetal  
Bovine Serum (FBS) and penicillin-streptomycin and were  
allowed to attach for an hour. The medium containing a  
15 higher concentration of cardiomyocytes that did not  
attach was transferred to a fresh 10 cm<sup>2</sup> dish and  
cultured at 37° C in 5% CO<sub>2</sub>.

The culture dishes had fibroblasts which were co-cultured  
20 with the cardiomyocytes. These fibroblasts were removed  
by repeated selective plating and by repeated complement  
fixation using an antibody, 1B10, (Sigma Chemical Co.)  
to the surface protein of fibroblasts (Singer et al,  
1989). This resulted in cultures with a very high  
25 percentage of cardiomyocytes. As expected, the primary  
cardiomyocytes obtained from fetal tissue underwent  
several more population doublings than those derived from  
adult heart tissue.

30 *SV-40 transformations (transforming a normal fibroblast  
cell line)*

Since the primary cultures stopped dividing, an indirect  
method was used to transfer the SV-40 gene in order to  
35 immortalize the cardiomyocytes. Used was a fibroblast  
cell line (DWFb1) transfected with a plasmid pNRS1, an  
SV-40 based mammalian vector, and depleted of its  
mitochondrial DNA (mtDNA) by treatment with ethidium  
bromide. DWFb1 was entirely dependent on glycolysis for  
40 energy requirements and was auxotrophic to uridine and

pyruvate. A few days after the final complement fixation step, DWFb1 cells were layered on the cardiomyocytes and allowed to attach for 4 hours at 37°C (fusing the  $\rho^0$  cells with primary cells in culture). The cells were  
5 fused with a 50% polyethylene glycol (PEG) solution for one minute, excess PEG was removed, the cells were gently rinsed in 10% dimethyl sulfoxide (DMSO) in culture medium, and subsequently grown under selection in  
10 uridine-free Dubecco's minor essential medium (DMEM) F-12 medium supplemented with 12.5% dialysed Fetal Bovine serum (FBS). This selection will eliminate the mtDNA-less cells ( $\rho^0$ ) cells that have not fused with the cardiomyocytes (selecting cells for a cell line).

15 Selection - The surviving hybrid cells were plated at low density and subcloned with glass cloning rings (establishing a clone/colony). About 40-50 colonies, each grown from a single cell, were picked from both adult, and fetal cardiomyocyte fusions. The clones were grown  
20 under selection, and screened for specific cell-type markers by immunocytochemical and molecular genetic analyses. Figure 1 describes schematically, the method used to generate our cardiomyocyte cell lines.

#### 25 *Immunocytochemical screening*

Cells from the clones plated on glass coverslips were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 1 hour, incubated with monoclonal  
30 antibody to SV-40 large T-antigen for 1 hour, and visualized by Texas Red conjugated secondary antibody. Clones that were positive for the large T-antigen were selected and screened immunocytochemically for the expression of muscle cell lineage markers, such as  
35  $\beta$ -myosin heavy chain, connexin-43, vimentin and desmin. The cells were incubated with the primary antibody for an hour at room temperature, and visualized with the appropriate secondary antibody conjugated with either FITC or Texas red. The cells were examined with a Zeiss  
40 fluorescent microscope using epi-illumination as

described and the images captured using SCION 1.60 image analysis software.

### *Histochemistry*

5

In order to evaluate mitochondrial function, succinic dehydrogenase (SDH) and cytochrome c oxidase (COX) histochemistry was performed. Cells plated on glass coverslips were air dried and incubated with the  
10 respective substrates, mounted on glass slides and studied with a Zeiss microscope, (King et al, 1992)

### *Molecular genetic analyses*

Total RNA was extracted from the clones using 'Totally  
15 RNA', an RNA isolation kit from Ambion. Reverse transcriptase PCR (RT-PCR) was performed using mRNA isolated from the clones with the following primers. The forward primer was located at nt 5735 - 5755 in exon 3 and the backward primer was located at nt 6333 - 6313 in  
20 exon 4. This primer set will amplify a 599 base pair fragment from the  $\beta$ -myosin heavy chain gene. RT-PCR was performed concurrently with the same samples using primers located within the cardiac muscle specific subunit of the cytochrome c oxidase gene (COX VIIc) as  
25 a control. The forward primer was located at nucleotide 1-24 (gcagagcttccagcggtat), and the backward primer was located at nucleotide 292-317 (catatgccatactagatatgtttgtc). The expected size of the amplified fragment is 300bp.

30

### *Electrophysiological studies*

The cardiomyocyte cell lines were grown on glass coverslips. Current recordings were performed by whole cell patch clamp electrophysiology. Under standard  
35 recording conditions, the resting membrane potential was recorded in all the selected cardiomyocyte clones. Dye coupling was performed by microinjection of Lucifer yellow into cells using a micromanipulator. The intracellular infusion of Lucifer yellow into the  
40 surrounding cells was observed and recorded.

## RESULTS

### *Generation of immortalized cardiomyocyte cell lines.*

Primary cardiomyocyte cultures from adult and fetal heart  
5 were repeatedly and selectively plated and complement  
fixed. This method eliminated most of the other cell  
types in the primary cultures and enriched for the  
cardiomyocyte content. By fusion with a  $\rho^0$  fibroblast  
10 cell line carrying the SV-40 gene and with selection that  
will permit the growth of only those cells that were  
fused, several growing colonies were observed. The  
parental DWFb1 cells were eliminated in the uridine-free  
medium, because they are respiration-incompetent. The  
15 unfused cardiomyocytes were terminally differentiated and  
did not survive passaging. Therefore, the surviving cells  
are fusion products of the cardiomyocytes and DWFb1,  
which were subsequently subcloned and more than 50 clones  
were isolated and cultured. The cells were passaged  
20 continuously in medium enriched with mitogens and growth  
factors for over 80 passages. At every stage of culture,  
several vials of cells were frozen in order to maintain  
stocks. These frozen stocks were found to be viable when  
thawed for regrowth.

### 25 *Characterization of cardiomyocyte cells*

#### *Immunocytochemical screening*

All 50 clones tested were positive for the large  
T-antigen by immunocytochemistry indicating that they  
were immortalized by the SV-40 gene carried by the DWFb1  
30 cells (Fig 2a). Six out of fifty adult cardiomyocyte  
clones and 14 out of fifty fetal cardiomyocyte clones  
expressed  $\beta$ -myosin heavy chain by immunostaining (Fig  
2b), and by RT-PCR, (Fig 3). Five representative clones  
that co-expressed the large T-antigen and  $\beta$ -myosin heavy  
35 chain (AC1, AC10, AC12, AC16 and RL14) were selected for  
further analysis.

The clones exhibited the same growth properties and  
tissue-specific markers, irrespective of whether they  
40 originated from the adult or the fetal tissue. The cells



expressed connexin-43, a cardiac tissue specific gap junction protein (Fig 2d). The cells did not stain with vimentin, data not shown), which is normally expressed by fibroblasts. However, they expressed desmin (Fig 2c), which is a muscle-specific marker, expressed by both skeletal and cardiac muscle.

#### *Molecular genetic analyses*

RT-PCR demonstrated the presence of  $\beta$ -myosin heavy chain mRNA which is specifically expressed by cardiomyocytes. COX VIIc which is a muscle-specific subunit of cytochrome c oxidase present in heart and skeletal muscle was also present in the cardiomyocytes (Fig 3).

#### *Electrophysiological studies:*

The cardiomyocyte-derived cell lines were examined briefly using patch clamp electrophysiology. Under standard recording conditions, the undifferentiated cardiomyocyte-derived cells were found to have stable, but low (-30 to -45 mV), resting membrane potentials. Consistent with their undifferentiated state, the cells lacked obvious voltage-activated conductances in whole-cell voltage-clamp recordings.

Intracellular infusion of Lucifer yellow revealed dye coupling of 3-6 cells per recorded cell, consistent with the presence of gap junction coupling between the cells. Presence of the gap junction protein connexin 43 was confirmed by immunocytochemistry.

#### **DISCUSSION**

Cardiomyocyte cell lines (AC1, AC10, AC12, AC16 and RL14) have been established from non-proliferating primary cultures derived from both adult and fetal heart tissues, vascular smooth muscle cells, skeletal myoblasts, neuronal cells or cardiomyocytes from myocardial DNA mutants by an unexpected novel and unique method that may be used with all primary cultures that have exited the cell cycle. Transfection of cells with vectors containing foreign DNA has been accomplished with cells.

This novel technique utilizes a mitochondrial function-based method to indirectly introduce the SV-40 gene into cells which are normally intractable to standard transformation techniques (Fig 1). First a normal fibroblast cell line was transformed with an SV-40 based mammalian vector to immortalize it. This cell line expresses the large T-antigen by immunocytochemistry. Subsequently, this cell line was treated with ethidium bromide to render it mtDNA-less,  $\rho^0$ , (King et al). The transformed  $\rho^0$  fibroblasts, ( $\rho^0$  DWFb1) are auxotrophic for uridine and pyruvate. They will grow only in medium supplemented with uridine and pyruvate since they are totally glycolytic and do not have a functional respiratory chain. By fusing postmitotic primary cells with  $\rho^0$  DWFb1 and by using a uridine-free selection that permits the growth of only those cells which have re-entered the cell cycle with normal respiratory chain function, immortalized human cardiomyocyte cell lines which still retain their endogenous mitochondrial DNA content are established herein. By employing suitable selection and subsequent subcloning steps followed by screening for cell lineage markers, clones were picked that express characteristics of adult human cardiomyocytes. By this protocol it may be possible to identify and characterize other clones based on the expression of specific markers of the different cell types that are present in the primary cultures and under the control of the nuclear genome.

The parental fibroblasts used for fusion (DWFb1) lack a functional respiratory chain, and therefore depend on glycolysis and uridine for their growth and survival. They are negative when stained for cytochrome c oxidase activity (COX). After fusion with cardiomyocytes, they have a fully functional respiratory chain activity as shown by the presence of normal cytochrome c oxidase (COX) activity, indicating the cardiomyocyte origin of mtDNA. The cell lines developed by this unique method have retained both the nuclear, and the mtDNA, from the cardiomyocytes. The fibroblast line were used as a

vehicle to transfer the SV-40 gene to immortalize the primary heart cells, when all other methods for transformation failed.

5 Efforts were focused to obtain cardiomyocyte-enriched primary cultures, by selective removal of fibroblasts. Therefore, our chances of generating relatively pure populations of transformed cardiomyocytes was high. As shown schematically in figure 1, our fusion and selection  
10 method favors the growth of only those cardiomyocytes that have fused with p° DWFb1 and contain the SV-40 gene. The unfused DWFb1 cells and the primary cardiomyocytes will not divide and grow under these culture conditions. The multinucleated cells were unstable and eliminated  
15 during subsequent passages.

Fifty large T-antigen positive subclones were selected and further screened immunocytochemically to characterize their tissue specific phenotype. Demonstrated in both the  
20 AC and RL clones was the presence of the  $\beta$ -myosin heavy chain and its mRNA as confirmed by RT-PCR.  $\beta$ -myosin heavy chain is expressed specifically by adult cardiomyocytes. All the cell lines expressed connexin-43, a major cardiomyocyte specific gap junction protein. Lucifer yellow, when  
25 microinjected into the cell, spread to the surrounding cells indicating that the gap junctions are open and the cells can communicate with each other. The cultures did not stain with antibody to vimentin, indicating that the cells are not controlled by the fibroblast nucleus. On  
30 the other hand, based on the staining with antibodies to desmin,  $\beta$ -myosin heavy chain, and connexin-43, the clones seem to be driven by the cardiomyocyte nucleus and express features characteristic of both cardiac and skeletal muscle. There is no evidence of any skeletal  
35 muscle in the tissue samples that were used to generate cell lines with both cardiac and skeletal muscle phenotypes. The skeletal muscle markers are perhaps expressed as a consequence of the transformation of the primary cardiomyocytes by the SV-40 gene resulting in  
40

their de-differentiation. Using the same reasoning, it may therefore be possible to induce these cardiomyocytes to differentiate in culture either towards the cardiac muscle or to the skeletal muscle lineage. These studies are currently being performed in the laboratory and may be a useful model in the study of both cardiac and skeletal muscle.

The cells exhibit a low resting membrane potential consistent with their undifferentiated state. Studies are currently being undertaken to determine if changes in their state of differentiation can result in changes in their electrophysiological characteristics.

Since heart cells are terminally differentiated they cannot respond to injury to heart muscle as occurs during heart attacks. The human cardiomyocyte cell lines described here may be useful for transplantation on damaged myocardium to repair and restore normal function. The potential use of these cells in cell transplantation techniques is important due to the presence of gap junctions and the cardiac muscle-specific gap junction protein, which play a role in cell communication. These cells when transplanted on heart tissue may potentially fuse with native myocardium, form a syncytium and functionally integrate.

Secondly, the cell lines provide a tissue culture model to evaluate pharmacological and physiological effects of drugs in order to devise and test therapeutic strategies. Cells generated by this method are evaluated as previously described by patch clamp electrophysiology, biochemical tests (eg. histochemistry) or molecular genetic analysis. The cells are then exposed by methods known to those skilled in the art, to a pharmaceutical preparation. After exposure the cells are again evaluated by the previously described methods. A comparison of the cells before exposure to the pharmaceutical preparation and after is indicative of the effectiveness of the preparation in enhancing or

inhibiting cell growth. This may be a useful model in studying biochemical and molecular genetic changes in cardiovascular disease as a result of the treatment with a pharmaceutical preparation.

5

Several point mutations of the mtDNA are associated with maternally inherited cardiomyopathy as the predominant or exclusive clinical feature. Using techniques developed and described herein, it is possible to deplete the cardiomyocyte cell lines of their endogenous mtDNA content and repopulate them with mtDNA harboring pathogenic mutations to evaluate the pathogenesis of maternally inherited cardiomyopathies, mitochondrial function or differences in the normal and pathological state of cells. This may reveal if there is a cardiac tissue specific phenotype that segregates with the cardiomyopathy specific mutations.

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This method of immortalization of post mitotic cells that have a finite lifespan in culture has a wide applicability to practically all cell types. This technology has been successfully utilized to generate not only human ventricular cardiomyocyte cell line but also vascular smooth muscle (VSM) cell line from human aorta and human skeletal myoblasts from muscle biopsies. Prior to this invention the primary VSM cells were unable to grow and divide beyond 14 passages, when they became senescent. After immortalization using the method disclosed in this invention cells have been successfully passaged over 50 generations and still continue to divide and grow. The skeletal myoblasts have also been passaged for 25 generations. Neuronal cells have also been successfully immortalized using this technique and are being characterized.

In summary, this invention developed human ventricular cardiomyocyte cell lines which can proliferate in culture through several passages, and can be repeatedly frozen, thawed, and propagated. The cells express both cardiac muscle and skeletal muscle-specific markers. When

cultured under suitable conditions, these pluripotent cells may be allowed to differentiate into either the cardiac muscle or the skeletal muscle lineage. They are potentially invaluable *in vitro* models to study differentiation of muscle in normal and pathological states with wide applicability to cardiovascular and neuromuscular research. This invention presents the first available human cardiomyocyte cell line of its kind. In general the methods taught in this invention can be applied to any primary cell culture that is post mitotic.

### **Example 2: Vascular Smooth Cells**

Human vascular smooth cells (VSM), from human aorta were immortalized by the same technique that was used for generating the proliferating cardiomyocyte cell lines. The VSM cells have a finite lifespan up to about 12 population doublings. These cells at passage 9 were fused with a mitochondrial DNA-less ( $\rho^0$  zero), fibroblast cell line, ( $\rho^0$  DWFb1) that carried the SV-40 gene, and was dependent on uridine for growth. Several clones were selected in medium containing no uridine. This medium permitted the growth of only the VSM cells that fused with the  $\rho^0$  DWFb1 cells. The parental DWFb1 cells were eliminated in selection. The unfused VSM cells eventually with advanced passaging were also eliminated.

These immortalized VSM cells express the large T-antigen, Fig. 4A, and have been passaged over 50 times. On screening for mitochondrial function, they exhibit normal succinate dehydrogenase activity, Fig. 4B, indicative of the VSM-origin of the mitochondria. The parental DWFb1 cells were  $\rho^0$  zero. We have done limited characterization of these transformed VSM cells. Further studies are necessary and are currently being performed in our laboratory.

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**What is claimed is:**

1. An immortalized human cardiomyocyte cell line.
- 5 2. An immortalized human vascular smooth muscle cell line.
3. The cell line of claim 1, wherein the cardiomyocyte cell line is designated AC16 (ATCC Designation No. PTA-1500).
- 10 4. The cell line of claim 1, wherein the cardiomyocyte cell line is designated AC10 (ATCC Designation No. PTA-1501).
- 15 5. The cell line of claim 1, wherein the cardiomyocyte cell line is designated RL14 (ATCC Designation No. PTA-1499).
- 20 6. The cell line of claim 1, wherein the cell line integrates functionally with normal or myopathic cardiac tissue as determined by measurement of syncytial beating of the tissue.
- 25 7. A method for treating damaged cardiac tissue in a subject which comprises transplanting the cell line of claim 1 into a subject's heart containing damaged cardiac tissue.
- 30 8. A method for preparing a human immortalized cell line derived from a post-mitotic primary cell culture which comprises:
  - 35 (a) providing a cell culture of human primary post-mitotic cells,
  - (b) providing a human fibroblast cell line which:
    - 40 (i) has been transfected with a replicable nucleic acid vector which immortalizes



the fibroblast cell line,

5 (ii) has been depleted of its mitochondrial DNA thereby rendering the fibroblast cell line subject to growth selection due incapacity to perform glycolysis;

10 (c) co-culturing the human fibroblast cell line of step (b) with the cell culture of step (a) under appropriate conditions so that cell fusion occurs; and

15 (d) growing the fused cells from step (c) in a selection medium which selects for cells with mitochondrial DNA,

20 (e) selecting cells from step (d) which contain a nucleus which originated from the cells of the primary culture, so as to prepare the human immortalized cell line.

25 9. The method of claim 8, wherein the cell culture of human primary non-proliferating cells in step (a) is a cell culture of primary human cardiac cells, primary human skeletal myoblast cells, human neuronal cells, or primary human osteoblast cells.

30 10. The method of claim 8, wherein the replicable vector is an SV-40 vector.

11. The method of claim 8, wherein the fibroblast cell line is designated DWFb1.

35 12. The method of claim 8, wherein the appropriate conditions for cell fusion in step (c) comprise incubation for about one minute in a 50% PEG solution.

40 13. A method for determining whether a composition of matter inhibits cardiomyocyte cell function which

comprises:

5 (a) admixing the composition with cells of an immortalized cardiomyocyte cell line prepared by the method of claim 8 in cell culture; and

10 (b) determining whether the cells in step (a) exhibit normal cardiomyocyte cell function by measuring gene expression or by measuring syncytial beating in culture, wherein decreased cardiomyocyte cell function indicates that the composition inhibits cardiomyocyte cell function.

15 14. A method for determining whether a composition of matter enhances cardiomyocyte cell function which comprises:

20 (a) admixing the composition with cells of an immortalized cardiomyocyte cell line prepared by the method of claim 8 in cell culture; and

25 (b) determining whether the cells in step (a) exhibit normal cardiomyocyte cell function by measuring gene expression or by measuring syncytial beating in culture, wherein increased cardiomyocyte cell function indicates that the composition enhances cardiomyocyte cell function.

30 15. The method of claim 13 or 14, wherein the composition of matter is a peptide or a peptidomimetic.

35 16. The method of claim 13 or 14, wherein the composition of matter is a small organic molecule.

40 17. The method of claim 13 or 14, wherein the composition of matter is a nucleic acid.

- 5      19.    The method of claim 18, wherein the carrier is a  
diluent, an aerosol, a topical carrier, an aqueous  
solution, an ionic solution, a nonaqueous solution  
or a solid support.

10

**IMMORTALIZATION OF  
HUMAN POST-MITOTIC CELLS**

5     **Abstract of the Disclosure**

10     The present invention provides an immortalized human  
cardiomyocyte cell line and an immortalized human  
vascular smooth muscle cell line. The present invention  
further provides a method for preparing a human  
15     immortalized cell line derived from a post-mitotic  
primary cell culture which comprises: (a) providing a  
cell culture of human primary post-mitotic cells, (b)  
providing a human fibroblast cell line which: (i) has  
20     been transfected with a replicable nucleic acid vector  
which immortalizes the fibroblast cell line, (ii) has  
been depleted of its mitochondrial DNA thereby rendering  
the fibroblast cell line subject to growth selection due  
to incapacity to perform glycolysis; (c) co-culturing the  
25     human fibroblast cell line of step (b) with the cell  
culture of step (a) under appropriate conditions so that  
cell fusion occurs; and (d) growing the fused cells from  
step (c) in a selection medium which selects for cells  
with mitochondrial DNA, (e) selecting cells from step (d)  
which contain a nucleus which originated from the cells  
of the primary culture, so as to prepare the human  
immortalized cell line.

FIG. 1

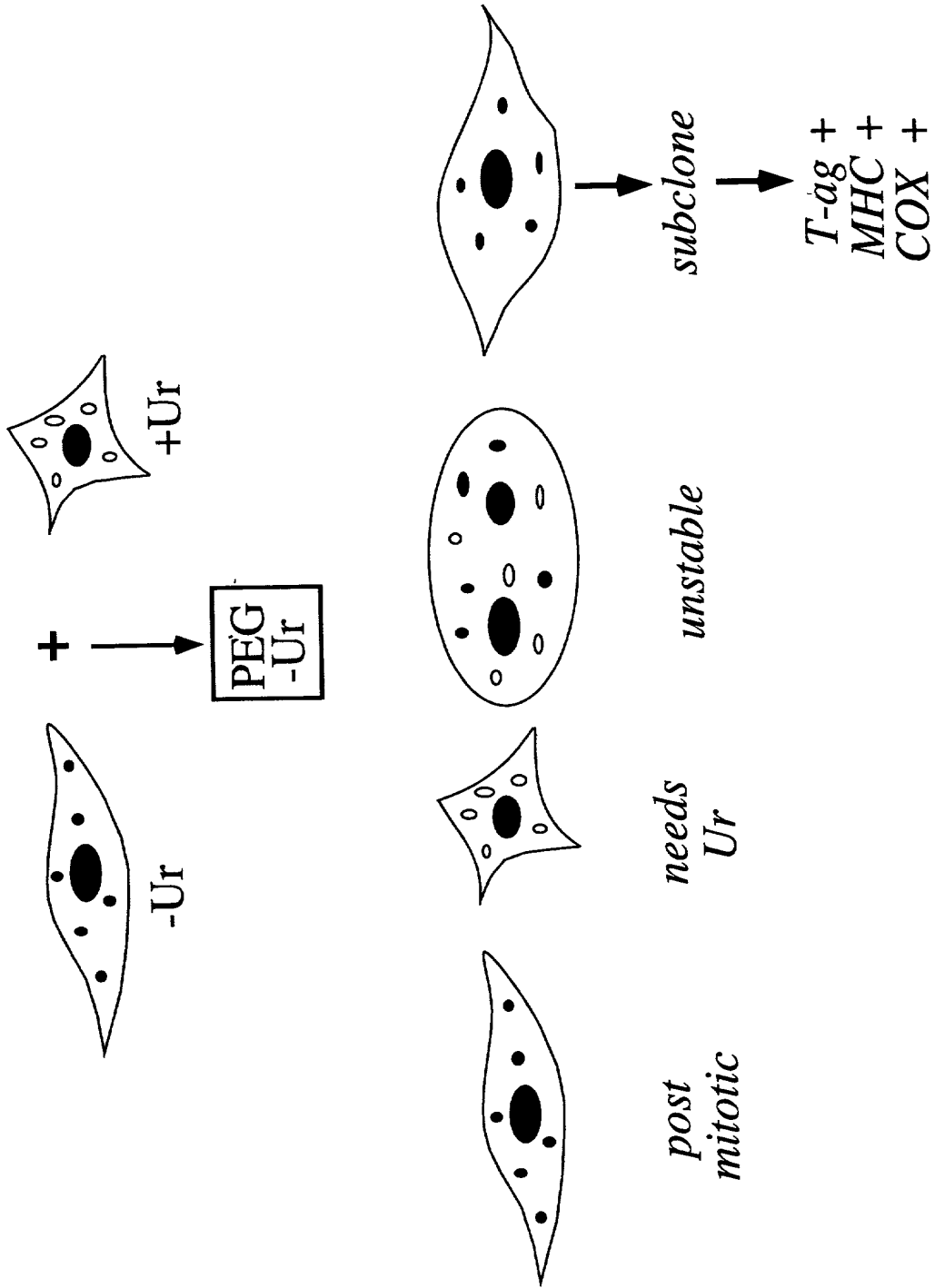


FIG. 2B



FIG. 2C

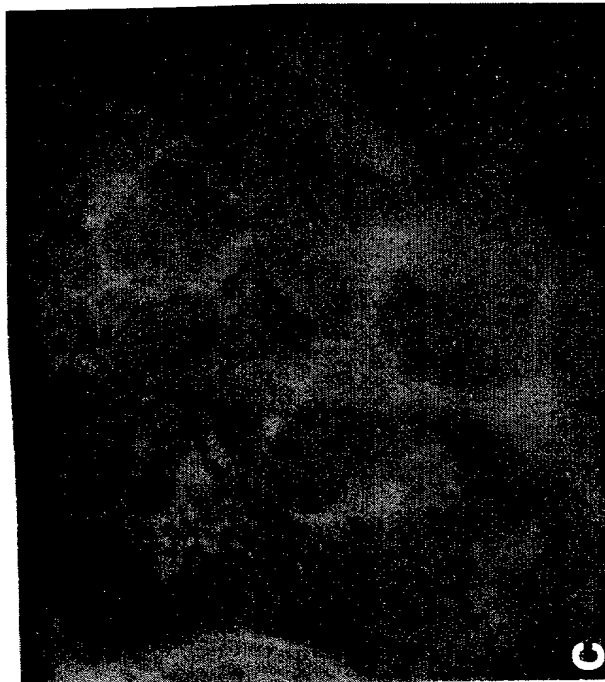


FIG. 2D



FIG. 3

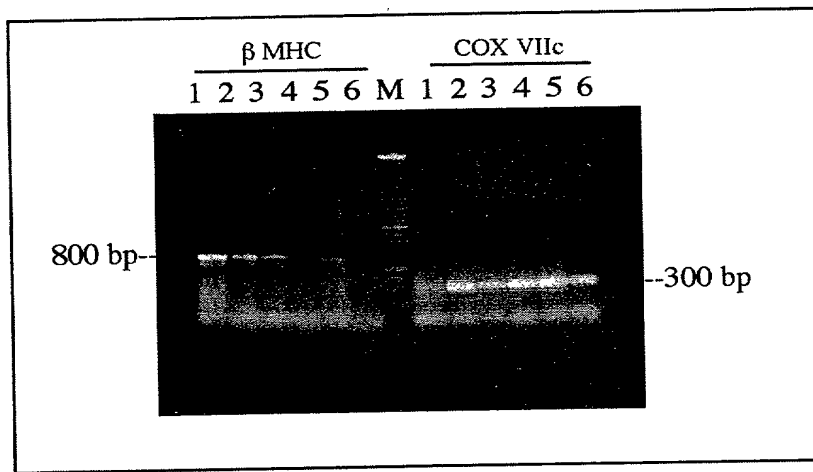




FIG. 4A

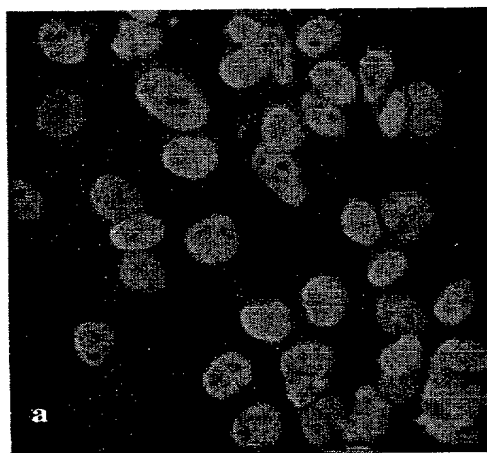
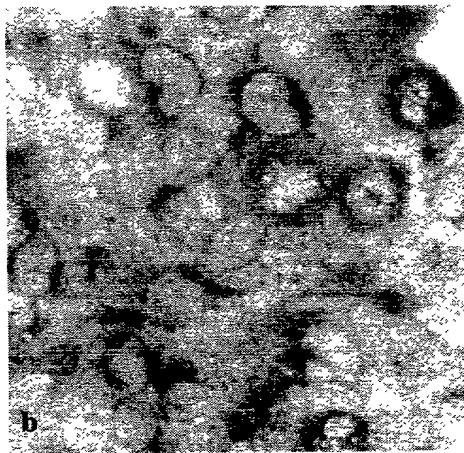


FIG. 4B



[illegible]

*My residence, post office address, and citizenship are as stated below next to my name.*

# IMMORTALIZATION OF HUMAN POST-MITOTIC CELLS

X is attached hereto.

X was filed on June 28, 2000 as

Application Serial No. Not Known Yet

and was amended \_\_\_\_\_ (if applicable)

*I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.*

*I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 (a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International Application which designated at least one country other than the United States, listed below. I have also identified below any foreign application for patent or inventor's certificate, or PCT International Application having a filing date before that of the earliest application from which priority is claimed:*

**Priority Claimed**

[illegible]

**Declaration and Power of Attorney**

Page 2

*I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below:*

<u>Provisional Application No.</u>	<u>Filing Date</u>	<u>Status</u>
N/A		

*I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States Application(s), or Section 365(c) of any PCT International Application(s) designating the United States listed below. Insofar as this application discloses and claims subject matter in addition to that disclosed in any such prior Application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56, which became available between the filing date(s) of such prior Application(s) and the national or PCT international filing date of this application:*

<u>Application Serial No.</u>	<u>Filing Date</u>	<u>Status</u>
N/A		

**And I hereby appoint**

John P. White (Reg. No. 28,678); Christopher C. Dunham (Reg. No. 22,031); Norman H. Zivin (Reg. No. 25,385); Jay H. Maioli (Reg. No. 27,213); William E. Pelton (Reg. No. 25,702); Robert D. Katz (Reg. No. 30,141); Peter J. Phillips (Reg. No. 29,691); Wendy E. Miller (Reg. No. 35,615); Richard S. Milner (Reg. No. 33,970); Robert T. Maldonado (Reg. No. 38,232); Paul Teng (40,837); Richard F. Jaworski (Reg. No. 33,515); Elizabeth M. Wieckowski (Reg. No. 42,226); Pedro C. Fernandez (Reg. No. 41,741); Gary J. Gershik (Reg. No. 39,992); Jane M. Love (Reg. No. 42,812); Spencer H. Schneider (Reg. No. 45,923) and Raymond A. Diperna (Reg. No. 44,063).

*and each of them, all c/o Cooper & Dunham LLP, 1185 Avenue of the Americas, New York, New York 10036, my attorneys, each with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent, to transact all business in the Patent and Trademark Office connected therewith and to file any International Applications which are based thereon under the provisions of the Patent Cooperation Treaty.*

Please address all communications, and direct all telephone calls, regarding this application to:

John P. White Reg No. 28,678

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*I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.*

Full name of sole or first joint inventor Mercy M. Davidson

Inventor's signature \_\_\_\_\_

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